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Jesse Ardosa
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Characterization of the Capsular Polysaccharides on Cell Membranes of Rhizobium trifolii and Three of Its Nod$^-$ Mutants

(TITLE)

BY

Jesse Ardosa

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

Master of Science in Chemistry

IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
CHARLESTON, ILLINOIS

1986
YEAR

I HEREBY RECOMMEND THIS THESIS BE ACCEPTED AS FULFILLING
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ABSTRACT

The ever-present specter of the inavailability of food production to increase in direct proportion to population growth haunts practically every nation of the world. As more and more tracts of land are incorporated to accommodate expanding cities, they become less available for food production. Emphasis, therefore, is placed on obtaining a higher yield per acreage planted. Since fertilization is not reliable in the long term due to an eventual depletion of raw materials for fertilizers, the ability of plants like legumes to assimilate free soil nitrogen on their own has become increasingly important [1,11,14,17]. However, the mechanisms involved are still not well-understood, so more research have to be done to make this the cornerstone of increasing plant productivity.

Indeed, the importance of understanding biological nitrogen fixation is accentuated by the number of researchers involved -- biochemists, plant physiologists, molecular geneticists, and biologists, to name a few. Countries around the world, from Australia to Japan to Sweden to Argentina, have research projects underway involving biological nitrogen fixation and/or plant productivity.

In our lab, the research focus is from both biochemical and immunological point of views. This particular work involves the study of the biochemistry of a cell surface macropolysaccharide -- the capsular polysaccharide or CPS. This is obtained from a pure culture of *Rhizobium trifolii*, a hac^+, nod^+, fix^+ Gram negative bacteria that could be found naturally in the soil. Together with its nod^- mutants, comparisons were done on the polysaccharide make-up of the CPS [25]. The CPS does not constitute a part of the cell membrane like the LPS

nor is it excreted freely into the media like the EPS. Rather, it adheres on the surface of the bacteria in the form of capsules [7,15].

From the isolated crude CPS, EPS-like materials called acidic CPS (ACPS) and LPS-like materials called neutral CPS (NCPS) were obtained upon treatment with CTAB. GC analysis revealed that the ACPS contains galactose and glucose whereas the NCPS has 2-O-methyl-6-deoxyhexose, fucose, mannose, galactose, 3-N-methyl-3-amino-3,6-dideoxyhexose, and glucose in both parent and mutant strains. KDO, acetyl groups, pyruvic acid, and uronic acid were present in both ACPS and NCPS of all strains using specific colorimetric assays.

Mild acid hydrolysis showed that the polysaccharide fractions of both ACPS and NCPS may be attached to a lipid, perhaps via KDO linkages. SDS-PAGE analyses showed different patterns of molecular aggregation between the ACPS and the NCPS but not within strains. Methanolysis of the samples revealed that the uronic acids in ACPS's are galacturonic acid while in NCPS's, they are both galacturonic and glucuronic acids.

These results suggest that the CPS of R. trifolii may have a dual character resembling that of E. coli and N. meningitaides, both Gram negative animal pathogens [24,33]. Whether the CPS in R. trifolii functions in the same manner still remains to be determined, as is its mode of attachment to the bacterial cell surface.

Lastly, the role of the acetyl groups merits careful investigation since these, together with heptose, correlate proportionally with phage-binding abilities [23]. Overall, the study of the CPS is still in a stage of infancy and a lot more research have to be done to determine whatever role it plays in the R. trifolii -clover symbiosis [7,15,35].

This work is specially dedicated to my parents, Mr. and Mrs. Alberto M. Ardosa, to whom my love and devotion is absolute, to my brothers Jun and Ruffy, and sisters Cecille, Beth, Dothy, Ellen, Gilda, and Doris, who made me proud to be a part of the family, and to my uncle and his children -- Sally, Femy, and Glenn, who form part of my extended family.

To Cecille, the only one who truly understands me; to Beth, who pushes me to be strong; to Dothy, the one who patiently puts up with me; to Ellen, who takes care of me all the time; to Gilda, who makes me feel like I really am a big brother; to Jun, the stranger I share the room with and call brother; to Doris, who cries a lot from my sometimes infantile jokes; and to Ruffy, my little bro who cries for me when I say goodbye -- I dedicate this work as a token of the love and appreciation that I have for you all as a brother.

ACKNOWLEDGMENTS

The preparation of this thesis would not have been easy without the help and encouragement of faculty members and fellow research workers. I gratefully acknowledge their patience and help all through those days when nothing else seemed to be working for me. The laughter that we have shared, the successes and failures that we have experienced and learned from each other, the sharing of materials, the secret deals, and, yes, the serious discussions of results and exchanges of ideas (there were times, too, when even the worst of us has to sit down and get serious) are moments that make all the works, disappointments, and sacrifices worth the effort.

Special thanks to Dr. Russell Carlson, my research adviser whose patience and guidance have made this thesis possible, to Brian Hanley (EPS) and Bob Shatters (LPS), the other corners of the triad in the three-pronged attack to unravel the mysteries of nitrogen fixation in clover, to Mrs. Steele, the repository of materials and methods that we used in our respective research, and to Dr. David Ebdon, friend, teacher, and confidant whose efforts as department chair has been my constant source of energy. This thesis is as much as theirs as it is mine.

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INTRODUCTION

Biological nitrogen fixation has grown in importance and has come under increasing scrutiny over the years due to the rapid depletion and subsequent increases in the price of the raw materials from which ammonia, the primary synthetic fertilizer used today, is manufactured. For instance, the well known Haber process for the synthesis of anhydrous ammonia consumes natural gas at an equivalent of three hundred million barrels of oil annually [1]. The amount of reactants converted into product per pass through the reaction chamber is rather low enough, around 15% at 150 atm and 350^{o}C, to make recycling necessary [34]. Of the total nitrogen fixed worldwide (around 175,000,000 metric tons per year), 69% is accounted for by biological means as compared to only 15% from nitrogenous fertilizers [1]. Indeed, if we search for a solution to the increasing pressure to improve plant productivity -- something that depends strongly on a delicate balance between soil nitrogen and plant growth -- biological nitrogen fixation is a noteworthy place to start.

Molecular nitrogen, as it is, is of no use to the plants but it can be reduced to ammonia [18], the form in which nitrogen is readily incorporated into the plants' biosystems. The reduction process that takes place is catalyzed by a nitrogenase reductase/nitrogenase enzyme complex and is believed to be

$$N_2 + 6\ Fd\ (red) + 12\ ATP + 12\ H_2O \text{ -----> } 2\ NH_4^+ + 6\ Fd\ (oxid) + 12\ ADP + 12\ Pi + 4\ H^+$$

with a ΔG = -136 kcal/mol nitrogen reduced [1].

However, this reaction does not just occur readily. Ferridoxin (Fd) and adenosine triphosphate (ATP) must be supplied by the plant as

low potential reductant and energy source, respectively. This poses a problem as to what increased nitrogen fixation could eventually cost the host plant.

Molecular nitrogen comes from the environment and in order to be reacted upon, it must first be immobilized, i.e., fixed. This is something that no living plant can do by itself. However, leguminous plants have the potential to fix molecular nitrogen biologically when infected by certain types of soil bacteria that possess the capability to fix nitrogen. This infection process is symbiotic in nature and is highly specific since it invariably proceeds through host-symbiont recognition. One such interaction involves clover as the host and Rhizobium trifolii, a species of bacteria belonging to the family Rhizobiaceae, as the symbiont.

The association of R. trifolii bacteria with the root hairs of clover follows a general pathway common to most Rhizobium -legume symbiosis [5]. This involves: (1) the adsorption of bacteria onto the root hairs of the host plant, (2) the recognition between symbiont and host, (3) the distinctive root hair deformation, (4) the formation of infection threads in root hairs, (5) the progression of bacteria, within the infection threads, into the cortical cells, and (6) the induction of nodular meristems. This is a progressive process whereby each step builds upon the other. The failure of any of these steps will prevent the subsequent steps from occurring.

The recognition between host and symbiont is a subject of lively discussion, especially its mode of attachment. In most recent reports, lectins, a group of proteins or glycoproteins with at least two sugar binding sites that precipitates glycoconjugates are often implicated in the attachment process [4,6]. In the case of R. trifolii -clover

symbiosis, lectins have been postulated to be a key element in the attachment of the symbiont to the host. However, in Rhizobium japonicum, a species of Rhizobia that specifically infects soybeans, certain available data refute this lectin-mediated recognition hypothesis [7]. The attachment of the bacteria onto the root hairs of the host legume leads to the formation of a sheath or capsule that eventually surrounds them. Following this, the root hairs undergo distinctive deformation or curling and trap the bacteria inside the resulting invagination. The sheathlike material surrounding the bacteria then begins to penetrate the root hair tissue forming infection threads through which the bacteria enter or invade the cortical cells. Upon successful completion of the first five steps, nodules start forming.

In the nodules, the invading bacteria assume a vegetative state by undergoing a series of physiological changes until they have become virtually a part of the root cells themselves [11]. They are called bacteroids and they behave just like the other cellular organelles and perform a well defined function. Although free living forms of Rhizobia can fix molecular nitrogen, once they become part of the root tissues as bacteroids, fixation takes on an entirely different form, i.e., through the combined action of the nitrogenase enzyme complex whose synthesis and activity are induced by the interaction of the bacteroids with the cell cytoplasm. These bacteroids have become nitrogen fixing organelles and are the sites of nitrogen fixation and reduction in the cell.

Of particular interest to our research group is Rhizobium trifolii, a species of bacteria of the genus Rhizobium that infects clover. This research is concerned with the bacterial species coded

ANU-843, a wild type parental strain, and three of its mutants, ANU-845, -851, and -871. Rhizobia are rod-shaped, monoflagellated, Gram-negative bacteria which may be classified into two groups according to their growth pattern: (1) fast growing, which has a generation time of 3 - 6 hours, and (2) slow growing, which has a generation time of 18 - 20 hours in a liquid culture medium [8]. Rhizobium trifolii belongs to the first group.

The parental or wild type strain, ANU-843, is capable of root hair curling (hac^+), nodule formation (nod^+), and fixing nitrogen (fix^+), whereas the mutants are defective at the root hair curling (hac^-) step. The strains were obtained from Dr. Barry Rolfe of the Australian National University. Our work is directed towards the characterization of the mutants relative to the parent strain and focuses on the isolation and comparison of the composition and structure of the macropolysaccharides in the cell wall of these bacteria. Three types of macropolysaccharides have been identified from the cell wall of R. trifolii -- (1) the extracellular polysaccharides (EPS), which the bacteria excrete freely into the growth media, (2) the capsular polysaccharides (CPS), which adhere closely onto the cell wall and form a coat around the bacteria, and (3) the lipopolysaccharides (LPS), which are legitimate components of the cell wall's outer membrane, the head (lipid) being embedded in the membrane and the tail (polysaccharide) sticking out to the cell environment.

The CPS, which is the focus of this research, is believed to be an EPS or a combinaton of both EPS and LPS and may not be present in all strains of Gram negative bacteria. Its origin is somewhat ambiguous and not as clearly defined as either the EPS or the LPS. Most data suggest that the CPS is part of the EPS or the EPS itself,

though why it adheres to the cell wall and forms capsule is still unknown. Since preliminary work in our laboratory indicated that the EPS and CPS are similar and that the hac$^-$ mutants lack CPS [8,10], further research was required to determine the relationship between the EPS and CPS from the parent and its mutants.

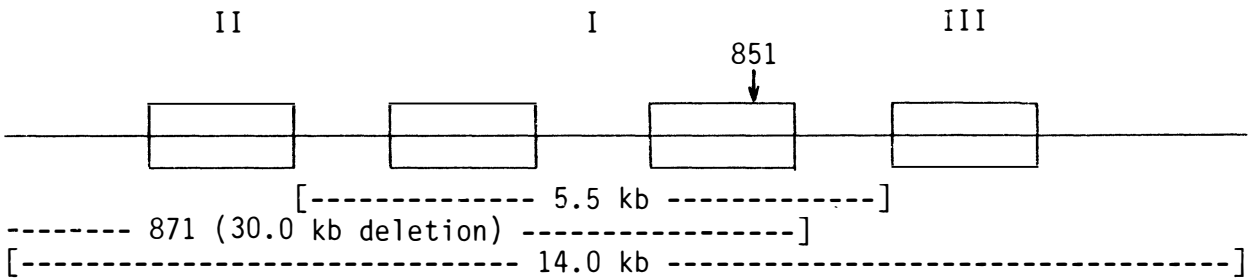
The mutants used in this work were obtained either by deletion of a certain fragment or fragments of the DNA or by insertion of a DNA fragment (transposon) into the genomic DNA. Deletion may be carried out by using endonuclease enzymes, by chemical mutagenesis, or by heat curing [11], and insertion by transduction whereby a plasmid that contains the transposon is transferred from one bacteria to another [12].

In the case of the mutants from ANU-843, both insertion and deletion methods were employed (personal communications, Dr. R. Carlson). Strain ANU-845, a heat-cured deletion mutant, has a large plasmid DNA missing. This plasmid contains all the genes responsible for symbiosis and is called the symbiotic (pSym) plasmid. Strain ANU-851, a pSym::Tn5 insertion mutant, has a piece of DNA (Tn5) inserted into the portion of the pSym plasmid of the parental strain that affects nodulation capabilities. Strain ANU-871, a deletion mutant of ANU-851, is missing a 30-kilobase region of the pSym plasmid (Figure 1).

By comparing the structures of the macropolysaccharides of the mutants to that of the parent bacteria, the results of these mutations on the production of macropolysaccharides would hopefully be found; such as an alteration of the sugar compositions and/or quantities; and whether or not these alterations are the cause of the different phenotypes of the mutants. In addition to these objectives, this

particular research project also hopes to gather information that would help establish the "true identity" of the CPS and if it has a function in the symbiotic process.

FIGURE 1. A region of the R. trifolii p-Sym plasmid showing where the mutations are. The plasmid is the region of the DNA where all the genes (hac, nod, nif) necessary to affect the early stages of symbiosis are located. The arrow points to the region where plasmid is inserted for ANU-851. Regions I, II, and III are designated in order of discovery.



EXPERIMENTAL

Basically, the research proceeded on a sequence of two general categories which can be classified as: (1) biological, and (2) chemical. This classification is purely arbitrary and is based on what was done and the principles involved and/or techniques applied in doing them. The biological category involves the growing and harvesting of bacteria and the isolation of the CPS. The chemical category covers the purification and determination of the composition of the CPS.

A. Biological Aspect of the Research

The first category, biological, employed bacteriological techniques in growing bacteria and in determining the presence of the transposon, Tn5, in the plasmid of the growing bacteria. Hardly any chemistry was done in this stage of the research.

The growing of bacteria [8,10] was done by inoculating four previously sterilized 250-ml flasks containing about 40.0 ml of fresh Modified Bergersen's (MB) media (see Appendix for composition) with a strain of R. trifolii. These flasks were placed on a shaker at room temperature, allowed to generate for four days, then transferred to a sterilized container containing 8 liters of fresh, sterile MB media and aerated with a stream of filtered air. The growth pattern was monitored twice each day by taking a 1 to 2-ml aliquot and reading its absorbance at 620 nm against deionized water. The bacteria were harvested when the absorbance read anywhere between 1.50 to 1.80, the late exponential stage of growth.

Contamination must be avoided in growing the bacteria so all the

instruments used have to be sterilized. Flaming techniques were employed in transferring the samples from one container to another and in taking aliquots for spectroscopic determination. Furthermore, checks were done at three different stages of growth: (1) prior to inoculation of 250-ml flasks with bacteria from the slant, (2) prior to the transfer of the 250-ml inoculum into the large container, and (3) prior to harvesting the 8-liter batch in the large container. The methods used were by Gram staining (for microscopic examination) and by streaking the bacteria on plates (for macroscopic determination of growing colonies) of nutrient agar (NA), MB media, and MB media + 100 ug of kanamycin/ml of the media, respectively. Normally, R. trifolii will not grow on NA and only ANU-851 will grow on media + kanamycin. Growing bacteria on a plate containing media + kanamycin serves a dual purpose -- to check for the presence of Tn5 in the pSym plasmid in addition to checking for contaminants. The Tn5 transposon codes for resistance to kanamycin.

Double-checking for contaminants is obviously essential such that the failure of one of the checks warrants the destruction of the whole batch. Only when these requirements are satisfied could harvesting of the bacteria proceed.

The harvesting of bacteria was done by centrifugation on a Sorvall SS-3 or SS-4 Manual Superspeed Centrifuge (used entirely throughout the harvesting unless otherwise specified) at 8,000 rpm (10,400 x g) for 15 - 25 minutes. This allowed the bacteria to form pellets at the bottom of the centrifuge tubes which were then collected, washed three times with 250.0 ml of fresh, sterile MB media to remove any EPS that may be adsorbed to the pellets, and separated by centrifugation. The media-washed pellets were collected, suspended in

physiologically buffered saline (PBS) solution (see Appendix for composition), blended in a Waring blender on and off at 1-minute intervals for 5 minutes, and centrifuged. The resulting supernatant liquid contains the CPS as determined by Anthrone assay (see Appendix for procedure) at 620 nm. The blending was repeated 5 times and the supernatant from each were pooled, concentrated in a rotary evaporator to about 200 ml, and centrifuged to remove extraneous matters. To the concentrate, 3 volumes of ethyl alcohol were added and the resulting precipitate was recovered by centrifugation. The precipitate was dissolved in deionized water, placed in a Spectrapor membrane tubing (used for dialyses all through this work) whose molecular weight cut-off is 12,000 - 14,000, and dialyzed against a bucket of deionized water for 3 days, changing the water twice daily. The dialysate was recovered, freeze-dried, and weighed as the crude CPS (CCPS).

B. Chemical Aspect of the Research

The second part of the research was categorized as chemical since it consisted of the purification of the CCPS, the chemical tests done to determine and quantify the monosaccharides, and the methods used to resolve the similarities and/or differences of the CPS with two of its macropolysaccharide analogs, the EPS and the LPS.

The CCPS materials obtained were purified by the addition of cetyltrimethylammonium bromide (CTAB), a cationic detergent that reacts with the acidic moiety of the CCPS and precipitates them out of the solution (see Appendix for complete procedure). By centrifugation for 30 minutes on a table top centrifuge, the acidic CPS (ACPS) was recovered as precipitate and the neutral CPS (NCPS) as the supernatant.

The ACPS was washed with 25.0 ml of deionized water twice, dissolved in 10% NaCl and reprecipitated with 50.0 ml of acetone. The precipitate was recovered by centrifugation, redissolved in 25.0 ml of 10% NaCl and dialyzed in fresh 10-liter 1% NaCl solution each day for two days, followed by plain deionized water for four days, changing the water twice each day. The dialysate was recovered and freeze-dried and weighed as the dry ACPS. The NCPS was made 10% in NaCl and dialyzed as described above for the ACPS. Occasionally, a precipitate may form. When this happens, the dialysate is centrifuged and both precipitate and supernatant recovered for freeze-drying.

From each strain of the purified acidic and neutral CPS, a 10-ml 1.0 mg/ml solution was prepared. From these, the sugar compositions were assayed, either by a gas chromatographic (GC) method in a Hewlett Packard 5890 gas chromatograph or by a spectrophotometric method using LKB Biochrom Ultrospec 4050 spectrophotometer. The sugars, 2-O-methyl-6-deoxyhexose, fucose, mannose, galactose, glucose, 3-N-methyl-3-amino-3,6-dideoxyhexose, and heptose were analyzed as their alditol acetates (see Acetylation Method, Appendix) by a GC method with inositol as the internal standard. The external standards used were rhamnose, fucose, xylose, mannose, galactose, glucose, and heptose. For quantitative analysis, the GC was interfaced to an Apple IIe computer. Area peaks were calculated by a software entitled "Chromatochart" purchased from IMI in State College, Penn. The remaining components, 2-keto-3-deoxyoctonate (KDO), acetyl groups, uronic acid, and pyruvic acid, were determined spectrophotometrically using specific tests for each (see Appendix for procedures). These tests, too, allowed for both qualitative and quantitative determination of the sugars for which they are specific.

A comparison was also made to determine how the components of the CPS, both acidic and neutral, migrate when electrophoresed in a polyacrylamide gel using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method (see Appendix). The LPS from the parent strain, ANU-843, and from a wild type Salmonella minnesota and its rough mutant, Ra-60, were used as standards. The resulting gel was stained using silver nitrate to determine carbohydrate bands (see Appendix for staining procedure).

The ACPS of ANU-843 and -845 were also subjected to mild acid hydrolysis (see Appendix) and the fractions recovered as polysaccharide were applied onto a G-50 Sephadex column with a bed volume of 138 ml. This collects 2-ml fractions at a flow rate of 0.2 ml/min. The fractions were tested for the presence of polysaccharides by the standard Anthrone assay (Appendix). The fractions that gave a positive result to the test were pooled, lyophilized, and dissolved in deionized water to make a concentration of 1.0 mg/ml. A portion of this solution was assayed for the presence of KDO and uronic acid and the rest was acetylated for GC analysis.

To determine whether the uronic acids present in each of the strains were present as galacturonic or glucuronic acids, a fraction of the original 1.0 mg/ml solutions was treated by methanolysis (see Appendix for procedure) and the resulting methyl glycosides were reduced with sodium borohydride, acetylated, and analyzed by GC.

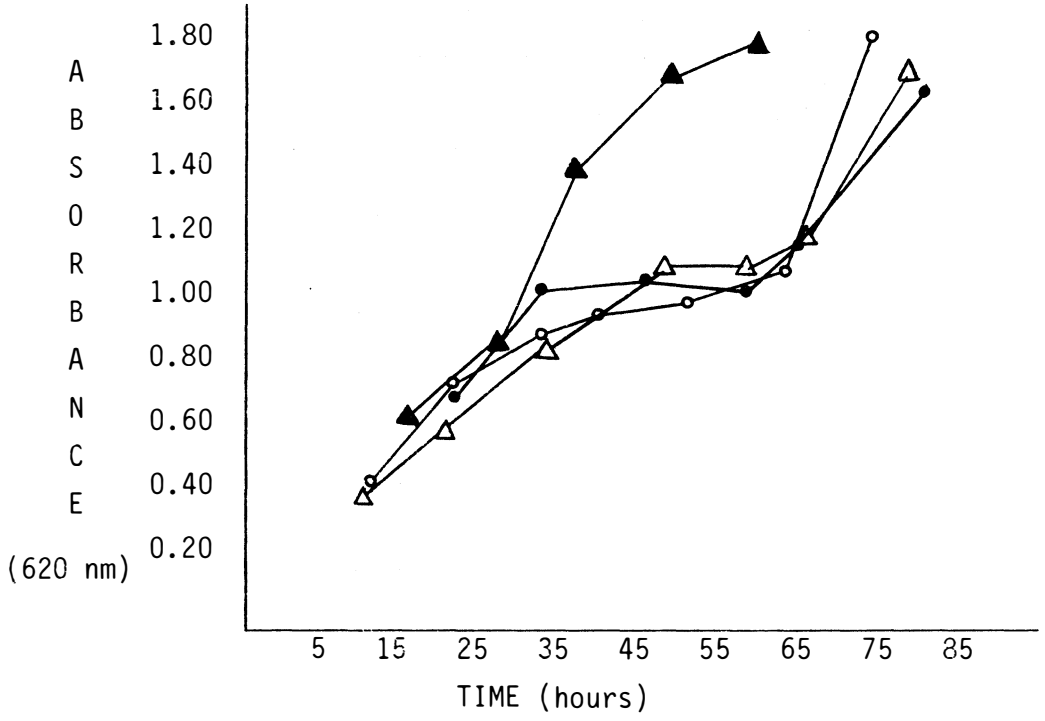
RESULTS AND DISCUSSION

Timing of the harvest and the purity of the rhizobial cultures and the polysaccharides have to be satisfied in the isolation and partial characterization of the CPS. Timing is important because the conditions and the stages of growth have to be the same for all rhizobial strains. The Rhizobia were harvested at the late exponential stage since it is here that bacterial growth reaches steady state and where changes in polysaccharides can occur [15]. This was determined graphically by plotting absorbance against time (Figure 2).

Purity have to be satisfied in: 1) the growing stage to avoid external contamination, especially from Gram positive bacteria, and 2) in the isolation of the CPS to avoid cross-contaminations due to the EPS and the LPS. It was noted in the first try that when CPS extraction was done up to seven times, the neutral fraction of the CPS increased a whole lot. This posed the question of whether or not, the excessive strain on the bacterial cell membrane caused by the blending of the pellets in the Waring blender to remove the CPS was enough to break loose the LPS and, hence, were extracted as CPS. Limiting the extraction up to five times minimized contamination from LPS and yielded enough CPS for viable compositional analyses (Table 1).

Treatment with CTAB separates the CCPS into EPS-like and LPS-like materials. This is possible since the EPS-like materials are more acidic in character due to increased substitution on its sugar molecules whereas the LPS-like materials have more of the neutral sugars. The cationic detergent, CTAB, preferentially reacts with the more acidic EPS-like materials and precipitates them out of the solution. To assume that some of the LPS-like materials could also be precipitated would not entirely be out of character since they, too,

FIGURE 2. Growth curves of Rhizobium trifolii



LEGEND: ▲ - ANU-843; △ - ANU-845; o - ANU-851; • - ANU-871

Table 1. Amount of CCPS obtained from each strains of bacteria grown in modified Bergersen's media.

Strain	843	845	851	871
Weight(g)	0.1804	0.1617	0.0670	0.0396

NOTE: Figures are in grams (dry weight)/8-liter batch bacterial culture harvested at the late stationary phase of growth.

Table 2. Result of the CTAB treatment of the CCPS of the different bacterial strains.

Strain	843	845	851	871
% Neutral Fraction (NCPS)	29.6	79.5	39.2	38.0
% Acidic Fraction (ACPS)	40.0	27.7	65.2	14.0

NOTE: Figures are in WEIGHT % of the amount of their respective CCPS treated.

possess acidic moieties. We could then anticipate contamination of the EPS-like materials by the LPS-like materials. To distinguish the precipitated CPS fraction from the one that remained in the solution, it is labeled acidic CPS (ACPS) and the supernatant fraction is labeled neutral CPS (NCPS). The result of this treatment is shown on Table 2.

The GC profiles of the NCPS fractions show the presence of the sugars 2-O-methyl-6-deoxyhexose, fucose, mannose, galactose, glucose, 3-N-methyl-3-amino-3,6-dideoxyhexose, and heptose whereas those of the ACPS's show only galactose and glucose (Table 3). The sugars 2-O-methyl-6-deoxyhexose, fucose, mannose, and sometimes heptose are present in the ACPS's in quantities too little to be quantified and are attributed to the presence of LPS and/or NCPS contaminants.

The sugar, KDO, which bridges the polysaccharide region and the lipid in the LPS was detected in the ACPS fraction of all strains. This could be interpreted either as further proof of contamination or as KDO being a part of the ACPS. In both LPS and NCPS, the amount of KDO present is not large enough for it to be considered part of the repeating units, only as linkage between the polysaccharide and the lipid portions [9]. The sugars heptose, 2-O-methyl-6-deoxyhexose, and fucose are present in both LPS and NCPS in more significant quantities (Table 3) than KDO. If, the KDO detected in the ACPS comes from contaminants such as NCPS and/or LPS, it would be logical to assume that the other sugars, heptose, etc..., would be detectable, too, perhaps even more so than KDO. However, since the GC profiles of the ACPS's from all strains show very negligible amounts of those sugars, it would be safe to assume that contamination is also negligible and that the KDO may not be from the contaminants.

Indeed, the presence of KDO in both the ACPS's and the NCPS's is

Table 3. Monosaccharide compositions of the neutral (N) and acidic (A) fractions of the CPS.

SUGAR	843N	845N	851N	871N	843A	845A	851A	871A
1) 2-O-methyl-6-deoxyhexose	4.38 ± .00	3.86 ± .02	4.08 ± .06	4.12 ± .16	0 0	0 0	0 0	0 0
2) Fucose	2.32 ± .10	1.92 ± .23	2.14 ± .17	2.23 ± .18	0 0	0 0	0 0	0 0
3) Mannose	4.50 ± .56	4.24 ± .23	4.28 ± .17	4.34 ± .28	0 0	0 0	0 0	0 0
4) Galactose	2.55 ± .69	2.82 ± .14	2.56 ± .30	2.51 ± .55	3.87 ± .91	2.75 ± .11	3.51 ± .32	3.15 ± .25
5) Glucose	4.62 ± .09	5.46 ± .18	4.96 ± .32	5.38 ± .23	17.51 ± 1.21	14.41 ± .66	17.89 ± 1.48	17.68 ± .63
6) 3-N-methyl-3-amino-3,6-dideoxyhexose	2.62 ± .40	2.77 ± .18	2.55 ± .42	2.78 ± .04	0 0	0 0	0 0	0 0
7) Heptose	6.99 ± .83	5.88 ± .65	6.30 ± .32	5.84 ± .40	0 0	0 0	0 0	0 0
8) 2-keto-3-deoxyoctonate	1.08 ± .12	0.88 ± .04	0.98 ± .10	1.00 ± .08	0.33 ± .04	0.30 ± .02	0.51 ± .03	0.62 ± .06
9) Acetyl Groups	1.46 ± .28	1.24 ± .20	1.39 ± .18	1.24 ± .16	1.42 ± .18	1.94 ± .54	2.17 ± .52	1.99 ± .56
10) Pyruvic Acid	1.85 ± .18	1.34 ± .30	1.30 ± .17	1.74 ± .36	7.71 ± .82	4.38 ± .28	4.35 ± .33	3.77 ± .39
11) Uronic Acid	11.51 ± .49	9.70 ± 1.27	11.93 ± .76	11.17 ± .52	26.41 ± 4.52	17.79 ± .74	17.61 ± 1.40	20.78 ± .85

NOTE: Figures are in WEIGHT % of the samples analyzed.

very interesting. It not only brings into focus the difference between the EPS and the CPS but also a suggestion that the CPS may be attached to a lipid fraction. The mild acid hydrolysis confirms this suggestion. If the KDO forms a bridge between the lipid and the polysaccharide fractions of the CPS, mild acid treatment would be enough to hydrolyze the keto linkage of KDO and separate the fractions. By extraction with a water immiscible solvent, such as chloroform or ether, the hydrophobic lipid fraction comes off in the organic layer while the hydrophilic polysaccharide fraction remains in the water phase. By the use of a Pasteur pipette, the layers could be separated and the fractions obtained either by freeze-drying (as in water layer) or by air-blowing using a gentle stream of filtered air (as in chloroform layer). This assay was done on ACPS's of ANU-843 and -845 and the NCPS and EPS of ANU-843 and the result is given on Table 4.

To compare the effect of mild acid hydrolysis on each polysaccharide, the water layers were chromatographed on a G-50 Sephadex column using unhydrolyzed counterparts as standards. The fractions that eluted from the column were assayed (ACPS's only) for neutral sugars using GC methods and for uronic acid and KDO using specific colorimetric assays.

The G-50 Sephadex column elution profile on Figure 3a shows that there is basically no difference in the unhydrolyzed molecules, all of which gave a response at the void volume only. In Figure 3b, the result of mild acid hydrolysis on the molecules is very pronounced. For both ACPS's, two responses were obtained, one at the void and the other near the included volume. The AEPS (included for purposes of comparison only) showed no change indicating that the treatment did not affect the molecule at all. The NCPS's gave responses which are almost

FIGURE 3a. G-50 Sephadex column profile of the acidic CPS (ACPS), neutral CPS (NCPS), and acidic EPS (AEPS) prior to mild acid hydrolysis. Standardization of the column was done with blue dextran (determines void volume) and glucose (determines included volume) standards.

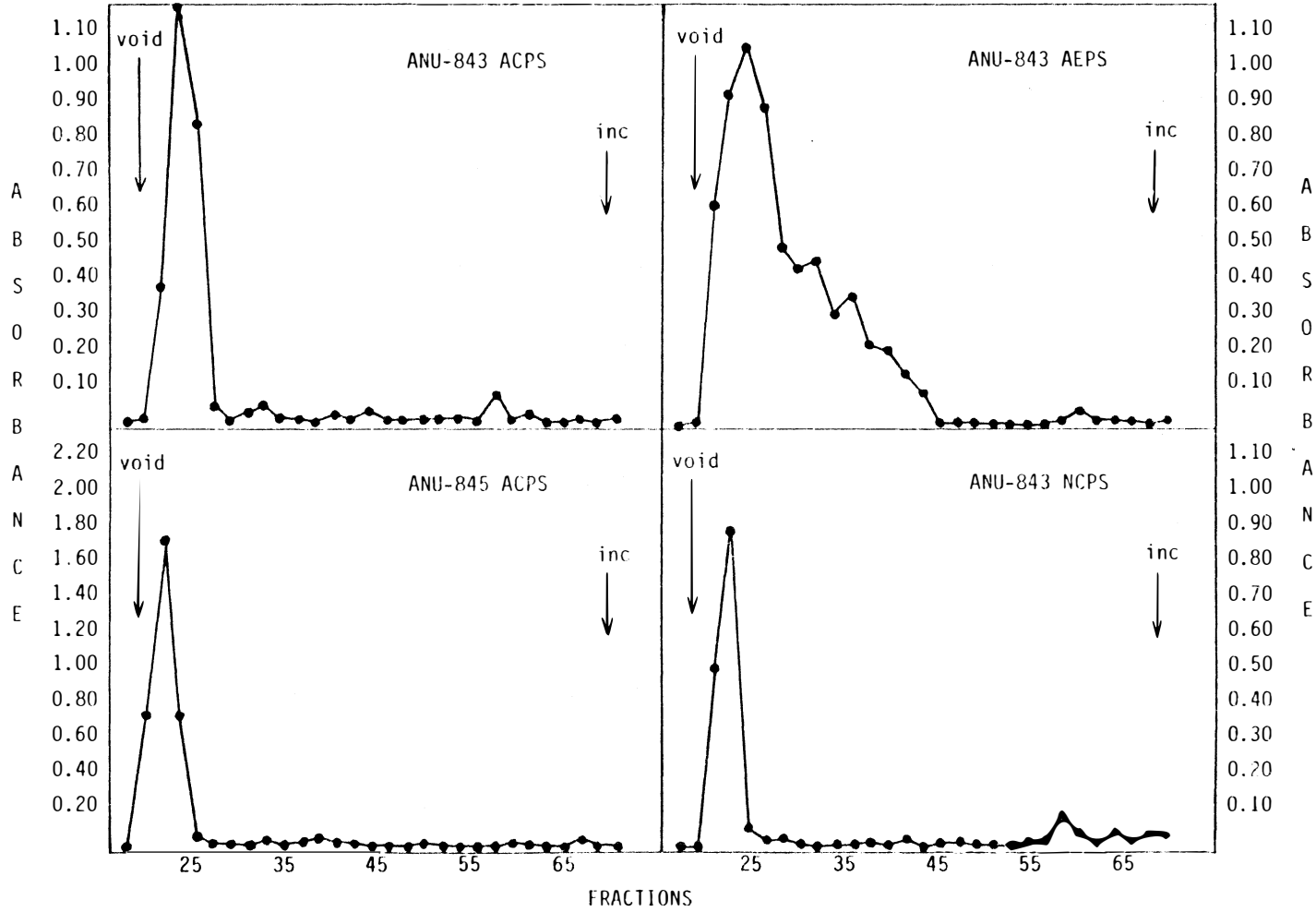


Table 4. Fractions obtained from mild acid hydrolysis treatment of representative polysaccharides, in weight %.

Polysaccharide	CHCl3 Soluble Fraction	H2O Soluble Fraction	Total@
843 ACPS	76.0 %	13.8 %	89.8 %
845 ACPS	85.7 %	6.6 %	92.3 %
843 AEPS	3.6 %	83.2 %	89.8 %
843 NCPS	31.2 %	59.6 %	90.8 %
843 LPS*	16.4 %	71.2 %	87.6 %

*Obtained from R. Shatters' research data on LPS's for his master's thesis, EIU, 1984.
@Refers to the total weight % accounted for, not of samples treated.

Table 5. Compositional analysis of the water-soluble fractions of the mild acid hydrolyzed polysaccharide samples of ACPS's after G-50 Sephadex column chromatography.

Sugar	843Void@	845Void@	843Inc@	845Inc@
Mannose	1.04 %	1.36 %	0.47 %	1.74 %
Galactose	6.33 %	4.66 %	1.17 %	4.77 %
Glucose	35.41 %	25.92 %	9.77 %	13.17 %
Uronic Acid	43.40 %	37.22 %	8.20 %	11.20 %
KDO	0.14 %	0.14 %	0.75 %	0.79 %
X*	8.12 %	7.86 %	8.74 %	9.88 %

*Unidentified signal, calculated by comparison with the signal due to xylose.
@Stands for the fractions that eluted at the void (Void) and the near included (Inc) volumes of the G-50 column.

identical to that of the LPS.

The appearance of a peak close to the signal due to xylose on the GC profile after mild acid hydrolysis of the ACPS's is rather unusual (Table 5). It is possible that this peak might be due to contaminants in the test tubes. Further work is required to ascertain the presence of this component and, if present, to identify it.

The result of the SDS-PAGE analysis (Figure 4) made it possible to compare the electrophoretic mobility of the components of both ACPS and NCPS with respect to LPS. In this comparison, EPS was left out since the conditions by which electrophoresis was done (15% gel, pH=8.8, 20 mAmp at room temperature) would not allow the EPS to migrate. This is due to its large molecular weight which makes it hard for the molecule to pass through the pores of the running gel (unpublished results of work done on EPS in our laboratory). The electrophoresis apparatus was set up such that at the top of the gel, the potential is negative while at the bottom, the potential is positive. By applying an electric current through the top, the polysaccharide samples, converted to their ionic forms in the slightly basic gel, move towards the bottom of the running gel, much like the migration of ions towards the positive and negative electrodes in electrolysis. The molecular weights of the aggregate fractions determine their electrophoretic mobility, the smaller ones being more mobile than the larger ones and migrate further down the gel.

From the silver-stained bands in the gel, it was determined that the pattern of NCPS migration closely resembled that of the LPS. The ACPS on the other hand, showed only two faint bands, possibly from the NCPS contaminants (Figure 4) because the amount of the ACPS samples used is twice that of the NCPS. This suggests that the aggregation of

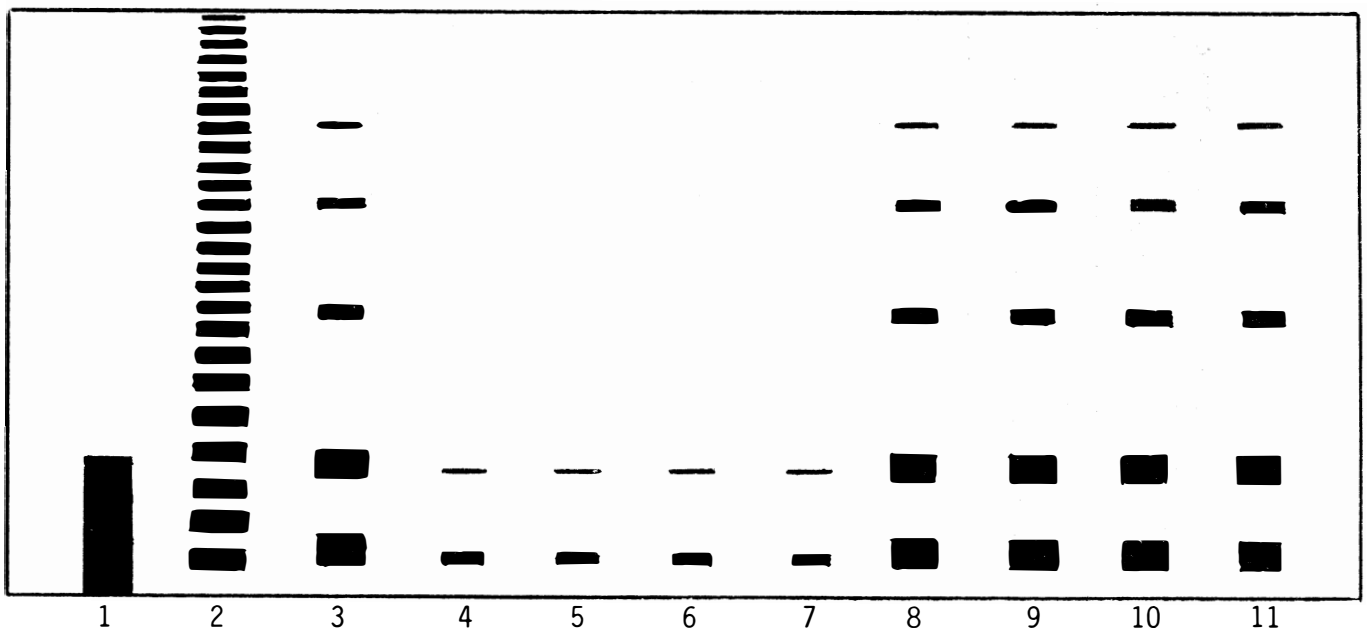


FIGURE 4. SDS-PAGE gel profile of the acidic and neutral CPS's and the standards. 1) Salmonella minnesota Ra-60, 2) Salmonella minnesota, wild type, 3) ANU-843 LPS, 4) ANU-843 ACPS, 5) ANU-845 ACPS, 6) ANU-851 ACPS, 7) ANU-871 ACPS, 8) ANU-843 NCPS, 9) ANU-845 NCPS, 10) ANU-851 NCPS, 11) ANU-871 NCPS. The first three wells are included as standards.

Total length of gel (vertical) is 9.50 cm and the distance of the bands (well #3) from top to bottom measures 3.20, 4.00, 5.15, 6.90, and 7.60.

S. minnesota Ra-60 is a mutant strain derived from S. minnesota, wild type.

Table 6. Monosaccharide compositions of the neutral (N) and acidic (A) fractions of the CPS after the reduction of uronic acid by methanolysis.

SUGAR	843N	845N	851N	871N	843A	845A	851A	871A
1) 2-O-methyl-6-deoxyhexose	4.01	3.58	4.04	4.18	0	0	0	0
2) Fucose	2.18	2.01	2.16	2.11	0	0	0	0
3) Mannose	4.66	4.57	4.61	4.75	0	0	0	0
4) Galactose	4.43	4.83	4.65	4.72	4.08	2.93	3.96	3.84
5) Glucose	6.58	6.47	6.01	6.91	29.13	19.42	23.30	24.10
6) 3-N-methyl-3-amino-3,6-dideoxyhexose	2.55	2.47	2.14	2.32	0	0	0	0
7) Heptose	6.67	5.68	6.33	5.70	0	0	0	0

NOTE: Figures are in WEIGHT % of the samples analyzed.

molecular fractions are pretty much the same for both the LPS and the NCPS but not for the ACPS. The ACPS fractions have smaller molecular weights and are, therefore, more mobile.

Methanolysis of the samples was done to reduce the uronic acids to their neutral hexose structures which, when acetylated, could be analyzed by GC methods. The formation of new signals corresponding to new sugars or the enhancement of existing signals will allow us to identify the uronic acids (see Table 6). However, the procedure was found to be good enough for qualitative purposes only. Signal enhancement was consistent. Those that showed marked increases were attributed to glucose in the ACPS's and to both glucose and galactose in the NCPS's (refer to Tables 3 and 6). Quantitatively, though, the reproducibility of the enhanced signal was poor. Perhaps, this was because the reaction that takes place is reversible. In the presence of alcohol and a strong acid, -COOH, the functional group in uronic acids, could be converted to an ester. But, under certain conditions, the hydrolysis of ester into acid + alcohol could go just as easily as the esterification of the acid group. If these two reversible reactions are competing, the chemical equilibrium will favor neither forward nor reverse reaction completely. Quantitative reproducibility of the results obtained will predictably be variable, hence, poor. No significant new signals were noticed except in one case where the mannose signal was increased considerably. This result could not be reproduced. It was concluded that the uronic acids in ACPS were glucuronic acids and in the NCPS, they are a combination of both glucuronic and galacturonic acids.

The presence of uronic acids in significant quantities is very interesting since this may help explain why the CPS adhere onto the

surface of the bacteria. It has been shown that polygalacturonates and polyglucuronates react with polyvalent cations, either as bidentate or monodentate ligands [26,27]. It is, therefore, possible that the CPS attachment to the bacterial cell wall is mediated by cations such as Mo(II), Fe(II and/or III), Ni(II), Mg(II), and Ca(II), which are present in the synthetic growth media and which occur naturally in the soil. These cations could provide a frame through which the CPS and the outer membrane could interact, through the -COOH groups of the uronic acids in ACPS's, and probably the phosphate of the phospholipid and the -COOH and $-NH_2$ of the proteins in the cell wall as the ligands. At present, no available data proves this type of interaction.

The presence of acetyl groups and heptose in the NCPS in reduced quantities may also be significant. These two sugars have been correlated to the phage binding ability of LPS in certain bacteria [23]. Pyruvic acid groups were also present in quantities whose proportion with respect to those in the LPS and the EPS of the parent and mutant strains is very interesting. There is a correlation in the amount of pyruvic acid between the NCPS and the LPS and between the ACPS and the EPS in the parent strain and none in the mutant strains. Due to lack of more information, it is probably sufficient at this point to just note these differences.

CONCLUSION

The data obtained from all the tests performed on the CPS proved to be, at best, informative but in no way conclusive. The pSym plasmid does not seem to carry the genes that code for the synthesis of the CPS, since even the mutant ANU-845 which lacks the entire pSym plasmid still produces CPS. Strains ANU-871 and -851 produce less CPS than ANU-845. Why this is so can not be explained by my results. Perhaps, this is because the genes in the p-Sym plasmid are regulatory in nature.

The presence of KDO in both the acidic and the neutral fractions of the CPS is quite interesting. In *R. trifolii* LPS, KDO is the sugar that forms a linkage between the lipid and the polysaccharide portions [9]. In *E. coli*, a Gram negative bacteria pathogenic to animals, KDO occurs in the CPS as a component of the repeating units of the polysaccharide chain [33]. Although the morphology and physiology of *E. coli* and *R. trifolii* may be different, an interesting comparison might be drawn from their CPS's. In *R. trifolii*, the CPS's have never been fully characterized simply because for a time, these molecules were considered to be a form of EPS. The presence of KDO, therefore, would be quite unusual based on the hypothesis that the CPS is a form of EPS. However, if we assume that the CPS is a different macropolysaccharide and compare it instead to *E. coli* CPS which has already been characterized [33], then the presence of KDO would not be so surprising. Even though *E. coli* infects animals while *R. trifolii* infects plants, specifically legumes, a valid comparison may still be drawn since the mode of infection has some parallel pathways. Infection by *E. coli* probably proceeds on a simpler pathway since it is

non-specific whereas that of *R. trifolii* is more complicated because of the constraint imposed upon by host specificity. However, both processes have to go through the stage whereby the bacteria attach themselves onto the host, penetrate through, and overcome the host's defense systems by "fooling" them. In *E. coli*, these steps are mediated by the CPS adhering onto the cell wall while in *R. trifolii*, the molecules responsible are basically unknown.

The *E. coli* CPS was shown to contain a lipid fraction attached to the polysaccharide chain by a phosphodiester linkage and, in some cases, the repeating units may contain KDO [33]. In *R. trifolii*, both lipid and KDO were detected in the neutral and the acidic fractions of the CPS (Table 3 and 4). From these data, we may hypothesize that the structure of the *R. trifolii* CPS may, in general, follow the pattern, lipid-phosphate-(KDO)n-polysaccharide as in CPS of *E. coli* or lipid-(KDO)n-polysaccharide as in LPS of *R. trifolii*. In this hypothesis, the presence of KDO is restricted to the linkage sites since its quantity is deemed insufficient for a component of the polysaccharide repeating unit. In fact, the presence of KDO and the lipid fraction is, so far, the strongest suggestive proof that the CPS is a distinct polysaccharide and not a form of EPS. However, this alone does not constitute conclusive identification since we still need more analyses.

In *E. coli* and *Neisseria meningitaides*, another animal pathogen that closely resembles *E. coli*, the host cells contain receptor sites on the surfaces of the cell membrane where the bacteria sort of "dock" [24,33]. From there, the pathogens either release their toxins which then penetrate into the host, all the while preserving the integrity of the cell membrane tissue, or cause tissue damage upon docking and

invade the interior of the host through these damaged sites [24,33]. The first is called enteropathogenic, the bacteria actually remain exterior to the host cells and only the released toxins penetrate through, while the second is called invasive, the bacteria enter into the host themselves. Once inside, the entering bacteria encounter a non-specific immunogenic response, the host's first line of defense. The specific immunogenic response comes only after the host cell recognizes the antigenic character of the invading substance and synthesizes an antibody specific to it. This antibody reacts with the invading substance (the reaction is termed opsonization, from the Greek which means "to prepare" as in food) rendering them vulnerable to phagocytosis [16]. All these actions are mediated by the CPS which provides the dual role of docking the bacteria onto the receptor sites in the host's cell membrane and, once inside, of evading the protective responses of the host cell. These actions correspond to a specific portion of the CPS molecule [24,33].

If we could visualize these processes and relate them to R. trifolii, then we may predict that it is the CPS, too, which is responsible for the infection of the host, and perhaps, the evasion of the host plant's defense responses. However, this host-symbiont interaction could have a mechanism entirely different from the animal pathogen system. The attachment of the symbiont onto the root hairs of the host legume is postulated to be mediated by lectin exuded by the plant root [6]. This lectin-binding hypothesis, though at present not entirely reliable due to a lot of conflicting data, is very promising. Since the symbiotic interaction is highly specific, it most probably involves mediation by something, not necessarily by lectin, rather than just the docking of the bacteria onto the host's receptor sites, as in

E. coli infection. The attachment process in symbiosis is expected to proceed via some sort of "opsonization" as in antigen-antibody interaction, only this time, the interaction takes place at the attachment site and not inside the host cell. Once this is accomplished, we may assume further that the mode of attachment is "invasive", causing root "tissue damage" and, as a response, root hair curling. This might be the difference between the parent and the mutants -- in its ability to inflict "tissue damage" leading to root hair curling (mutants are defective in the root hair curling, hac$^-$, step).

The idea of the CPS being a key molecule playing a very important role in symbiosis is both promising and appealing. The CPS molecule, by far, is the only molecule that was not shown to fail in its capability to cause root hair curling [35]. From the work in our laboratory, it is also the only molecule with a dual character. We may be able to presume that each fraction performs distinct functions such as the attachment of the bacteria onto the root hairs and the evasion of the host's defense responses just long enough to allow the bacteria to undergo some physiological changes and transform themselves to bacteroids.

The data obtained from my research do not allow me to reach a definitive conclusion as to why the parental strain is hac$^+$, nod$^+$, and fix$^+$ while the mutants are hac$^-$. Certainly, from the composition and quantity of the sugars present, there is not much of a difference among the strains. More analysis, especially structural, may be appropriate. Perhaps, the difference between the parent and the mutants may not even be in the quantity or the structure of CPS but in whatever regulates its adherence and/or location (personal communications, Dr. R.

Carlson).

From the above hypotheses, we may conclude that the CPS molecules of R. trifolii are implicated in the early stages of infection. However, as indicated, the events being compared -- infection processes, host responses, etc... -- are from two different biological systems, plant host-symbiont and animal host-pathogen systems. These systems involve two entirely different bacteria whose only common feature being that both are Gram-negative. These hypothetical conclusions, derive from parallel events that take place under different conditions and/or environment should be viewed only as a likely possibility, not a functioning reality in the R. trifolii -clover symbiotic process. Until more exhaustive studies are done, especially in the chemical and structural arrangements of the sugars in the CPS, a definitive conclusion should be deferred and unless these studies yield uncompromising proofs, the mediators of the infection process will remain a subject open to more discussion and research.

BIBLIOGRAPHY

1) Karel R. Schubert. The Energetics of Biological Nitrogen Fixation. Department of Biochemistry, Michigan State University. (1980).

2) G. Ya. Zhiznevskaya, I. N. Andreeva, A. A. Tibilov, and V. V. Il'yasova. Participation of Molybdenum in Formation and Functioning of the Symbiotic Nitrogen-fixing in Sea Buckthorn Nodules. Soviet Plant Physiology, No. 1-3, 30: 79-84. (1983).

3) Bernard J. Carroll and Peter Gresshoff. Nitrate Inhibition of Nodulation and Nitrogen Fixation in White Clover. International Journal of Plant Physiology, 110: 77-88. (1983).

4) E. Yu. Markov and E. E. Khavkin. Plant Lectins: Hypothetical Functions. Soviet Plant Physiology, No. 4-6, 30: 633-645. (1984).

5) J. M. Vasse and G. L. Truchet. The Rhizobium -Legume Symbiosis: Observation of Root Infection by Bright Field Microscopy After Staining with Methylene Blue. Planta, No. 6, 161: 487-489. (1984).

6) Bjorn Solheim. Possible Role of Lectin in Binding Rhizobia to Host Roots. Reprint from Lectins, III: 539-547. (1983).

7) Russell W. Carlson. Surface Biochemistry. Nitrogen Fixation: Rhizobia, 2: 199-234. (1982).

8) Duh-Jao Lin. Master's Thesis, Eastern Illinois University. (1983).

9) Robert G. Shatters. Master's Thesis, Eastern Illinois University. (1984).

10) Elroy Turnball. Master's Thesis, Eastern Illinois University. (1983).

11) J. J. Child. Nitrogen Fixation by Free-living Rhizobium and its Implication. Recent Advances in Biological Nitrogen Fixation, 1: 325-343. (1980).

12) Lubert Stryer. Biochemistry. 2 nd Edition. (1981).

13) G. N. Troitskaya, N. A. Suvrova, A. I. Obukhov, and G. Ya. Zhiznerskaya. Activity of Vitamin B12 Synthesis in Root Nodules of Soy and Other Legumes as a Function of the Level of Cobalt Accumulation in Them. Soviet Plant Physiology, No. 1-3, 30: 231-236. (1983).

14) G. J. Waughman, J. R. J. French, and K. Jones. Nitrogen Fixation in Some Terrestrial Environments. Nitrogen Fixation: Ecology, 1: 104-134. (1981).

15) D. A. Powell. Structural, Solution Properties, and Biological Interactions of Some Microbial EPS. Microbial

Polysaccharides and Polysaccharases, 1: 117-160. (1979).

16) Alastair Cunningham. Understanding Immunology. (1978).

17) Samuel Aldrich. Nitrogen in Relation to Food, Environment, and Energy. Special Publication 61, Agricultural Experiment Station, College of Agriculture, Univ. of Illinois at Urbana-Champaign. (1980).

18) K. T. Shanmugam, S. T. Lim, S. S. M. Hom, D. B. Scott, and H. Hennecke. Redox Control of Nitrogen Fixation and Conservation of Fixed Nitrogen, 17: 79-84. (1981).

19) Shlomo Hestrin. The Reaction of Acetylcholine and Other Carboxylic Acid Derivative with Hydoxylamine and its Analytical Application. J. Biological Chem., 180: 249-261. (1949).

20) T. V. Bhuvaneswari and B. Solheim. Root Hair Deformation in the White Clover- R. trifolii Symbiosis. Physiologia Plantarum, Fasc. 1, 63: 25-34. (1985).

21) B. Solheim and K. E. Fjellheim. Rhizobial Polysaccharide-degrading Enzyme from Roots of Legumes. Physiologia Plantarum, Fasc. 1, 62: 11-17. (1984).

22) G. Bradley, C. C. Gaylarde, and J. M. Johnston. A Selective Interaction Between Fe(II) Ions and LPS in P. vulgaris. J. of Gen. Microbiology, 130: 441-444. (1984).

23) Elzbieta Zajay and Z. Lorkiewicz. Role of O-acetyl Groups in the LPS Receptor for Rhizobium Phage 1-P. J. of Gen. Microbiology, 129: 3171-3176. (1983).

24) J. McLeod Griffiss and Brenda L. Brandt. Immunological Relationship Between the CPS of Neisseria meningitaides Sero Groups Z and 29E. J. of Gen. Microbiology, 129: 447-452. (1983).

25) Ann M. Hirsch, Daniela Drake, Thomas Jacobs, and Sharon Long. Nodules are Induced on Alfalfa Roots by Agrobacterium tumefaciens and R. trifolii Containing Small Segments of R. meliloti Nod Region. J. of Bacteriology, No. 1, 161: 223-230. (1985).

26) C. Gessa, M. L. de Cherchi, and A. Dessi, S. Deiana, and G. Micera. The Reduction of Fe(III) to Fe(II) and V(V) to V(IV) by Polygalacturonic Acid: A Reduction and Complexation Mechanism of Biochemical Significance. Inorganic Chemica Acta (Bioinorganic Chem. Letters and Articles), No. 4, 80(B8): 153-155. (1983).

27) S. Deiana, L. Erre, G. Micera, P. Piu, and C. Gessa. Coordination of Transition Metal Ions by Polygalacturonic Acid: A Spectroscopic Study. Inorganic Chemica Acta (Bioinorganic Chem. Letters and Articles), No. 6, 46(B1): 249-253. (1980).

28) John L. Middlebrook and Rebecca B. Dorland. Bacterial Toxins: Cellular Mechanisms of Action. Microbiological Reviews, No. 3, 48: 199-221. (1983).

29) Russell W. Carlson. Heterogeneity of Rhizobium Lipopolysaccharides. J. of Bacteriology, No. 3, 158: 1012-1017. (1984).

30) N. Blumenkrantz and G. Asboe-Hansen. A New Method for the Quantitative Determination of Uronic Acid. Anal. Biochem., 54: 484. (1973).

31) H. Katsuki, T. Yoshida, C. Tanegashima, and S. Tanaka. Improve Direct Method for Determination of Keto Acids by 2,4-dinitrophenylhydrazine. Anal. Biochem., 43: 349-356. (1971).

32) A. Weissbach and J. Hurwitz. The Formation of 2-keto-3-deoxyheptonic Acid in Extracts of E. coli. J. of Biological Chem., 234: 75. (1958).

33) K. Jann. Capsular Polysaccharide of Uropathogenic Escherichia coli. Bacterial Lipopolysaccharides, ACS Symposium, Series 231. 171-191. (1983).

34) Joseph LeBlanc, Jr., S. Madhavan, Raymond E. Porter. Ammonia. Kirk-Othmer Encyclopedia of Technology, 3 rd Edition, 2: 470-515.

35) Sean E. Ervin and David H. Hubbell. Root Hair Deformations Associated with Fractional Extracts from Rhizobium trifolii. Applied and Environmental Microbiology, No. 1, 49: 61-68. (1985).

APPENDIX

CRUDE CPS PURIFICATION PROCEDURE

PROCEDURE:

1) Dissolve 25.0 mg of crude CPS in 50.0 ml of 0.01 M sodium sulfate (Na_2SO_4) solution.

2) Prepare a 3% (w/v) solution cetyltrimethylammoniumbromide solution (CTAB) solution. Add 5.0 ml of this solution to # 1 dropwise with stirring. Solution may turn cloudy.

3) Place the solution on a shaker and shake at 37°C for 2 hours.

4) Collect both precipitate and supernate and treat them according to the following procedures:

To the precipitate:

1) Wash precipitate collected from # 5 with 25.0 ml of deionized water twice.

2) Dissolve the precipitate in 25.0 ml of a 10% NaCl solution and precipitate the polysaccharides by adding 25.0 ml of acetone. Centrifuge.

3) Collect and dissolve the precipitate in 25.0 ml of 10% NaCl solution and dialyze against 10.0 liters of 1% NaCl solution two times. Use a fresh 1% NaCl solution each time.

4) Dialyze against 10.0 liters of deionized water for four days, changing the water twice daily. Freeze-dry and weigh as the acidic CPS.

To the supernatant liquid:

1) Add NaCl to make the solution 10% in NaCl (w/v).

2) Dialyze as above. If precipitate forms, collect and dissolve in deionized water. Freeze-dry both precipitate and supernatant and weigh. The supernatant will be the neutral CPS.

NOTE: If the dialysis of the supernate produces precipitate in quantities large enough for viable analysis, check for neutral sugars using GC methods.

ACETYLATION PROCEDURE
(Preparation for GC Analysis)

NOTE: Standard sugars and samples must be dried in the vacuum oven prior to use in preparing the 1.0 mg/ml solutions.

PROCEDURE:

1) Prepare a standard sugar solution containing 1.0 mg/ml of each standard sugar (rhamnose, fucose, xylose, mannose, galactose, glucose, and heptose). This solution may be stored in the freezer and re-used.

2) Prepare a 1.0 mg/ml solution of inositol to use as the internal standard. This may be stored in the freezer and re-used.

3) Prepare a 1.0 mg/ml solutions of the sample polysaccharides.

4) Determine the % hexose of the samples using the anthrone assay.

5) Place a volume of sample equivalent to not more than 250 µg of hexose in a screw top test tube. In another screw top test tube, place 100 µl of the standard sugar solution. Add 20 µl of inositol to each tube and dry them by using a gentle stream of filtered air or by freeze-drying.

6) Add 500 µl of a 2.0 M trifluoroacetic acid (TFA) solution to each tube. Seal with Teflon-lined screw cap and heat at 121°C for 2 hours.

7) Remove tubes and blow-dry with filtered air gently. This may be done at 40 - 50°C unless your sample is a methylated polysaccharide which should be done at no more than 35°C.

8) Prepare a 10.0 mg/ml solution of sodium borohydride (or sodium borodeuteride) in 1.0 M ammonium hydroxide. For methylated samples, always use sodium borodeuteride. Add 250 µl of this solution to each sample, mix, and allow to stand for 1 hour (2 hours for methylated samples) at room temperature.

9) Add 50 µl of glacial acetic acid. Vigorous bubbling should take place! Repeat 2 more times.

10) Add 500 µl of 9:1 methanol (MeOH):acetic acid (HOAc) to each tube. Blow-dry gently using filtered air at 40 - 50°C (no more than 35°C for methylated samples). Repeat this step 4 times.

11) Add 500 µl of MeOH to each tube and blow-dry as in step 10. Repeat 4 more times.

12) Add 50 µl of pyridine and 50 µl of acetic anhydride to each tube. Mix, seal with Teflon-lined screw caps, and heat at 121°C for 30 min.

13) Cool on ice and extract by adding 500 µl of water and 500 µl of chloroform. Mix, centrifuge on table top centrifuge, and add another 500 µl of chloroform to this water layer. Collect both chloroform layers and blow-dry with a gentle stream of filtered air at room temperature.

14) Analyze by dissolving sample in 20 - 50 µl of dichloromethane and injecting 1 - 5 µl into the GC.

NOTE: To insure purity of all solvents, they should be spectrophotometric or HPLC grade or distilled prior to use.

****

ANTHRONE ASSAY FOR HEXOSE

REAGENTS:

(A) 0.2% anthrone in concentrated H_2SO_4
(B) 1.0 mg/ml of sample solution
(C) 1.0 mg/ml solution of glucose (standard)

PROCEDURE:

(1) Prepare a series of dilutions of the sample and bring them to a final volume of 500 µl using deionized water if necessary.

(2) Add 1.0 ml of reagent A to each tube. Vortex.

(3) Heat the tubes in a boiling water bath for 5 min and read the absorbance at 620 nm.

NOTE: For qualitative assay where samples contain a lot of sugars, step 3 may be omitted.

ASSAY PROCEDURE FOR KDO [32]
(2-keto-3-deoxyoctonic acid)

REAGENTS:

(A) 0.4 N H_2SO_4
(B) 0.04 N HIO_4
(C) 2% sodium arsenite ($NaAsO_4$) in 0.5 N HCl
(D) 0.3% thiobarbituric acid

PROCEDURE:

1) Put 200 µl of the samples in test tubes. Add deionized water to get this final volume if necessary.

2) Add 20 µl of reagent A to each sample. Vortex after each addition. Heat in a hot water bath for 30 min.

3) Add 250 µl of reagent B to each sample. Vortex after each addition. Allow to stand at room temperature for at least 40 min -- a precipitate may form.

4) Add 500 µl of reagent C to each sample. Vortex after each addition. Allow to stand at room temperature for at least 5 min after the last tube is filled. Sample turns dark brown and slowly clears on standing.

5) Add 2.0 ml of reagent D to each sample. Vortex after each addition. Place in hot water bath for 20 min.

6) Read the absorbance immediately at 548 nm. If precipitate forms as the solution cools, centrifuge for 1 min on a desk top centrifuge prior to reading the absorbance.

NOTE: Quantitative assay is done using a 0.1 mg/ml solution of KDO as standard and proceed as in hexose assay.

PYRUVIC ACID ASSAY [31]

REAGENTS:

(A) 500 µM of 2,4-dinitrophenylhydrazine (DNP), mol. wt. = 198.14, in 100 ml of 2.0 N HCl. Dissolve at 40°C. This remains stable for two weeks only.
(B) 2.2 N NaOH
(C) 2.0 N HCl
(D) 0.1 mg/ml pyruvic acid (used as standard)
(E) toluene
(F) 10% Na_2CO_3

PROCEDURE:

1) Prepare 200-µl samples in screw top test tubes. Use deionized water to bring the volume up to 200 µl if necessary. If samples contain protein, they should be deproteined with perchloric acid before proceeding to the next step.

2) Add 0.3 ml of reagent C to each tube. Seal with Teflon-lined screw caps and heat at 100°C for 3 hours.

3) Add 0.1 ml of reagent A to each tube. Vortex. Let mixtures stand for 30 min at room temperature.

4) Add an equal volume, 0.6 ml, of reagent E to each tube. Vortex. Remove bottom layer and add 0.6 ml of reagent F to the top layer (toluene layer). Vortex and remove top layer.

5) Add 0.4 ml of deionized water and 1.0 ml of reagent B to bottom layer of each tube from step 4. Vortex and read absorbance at 416 nm.

NOTE: If the samples give a colored substance not produced by DNP, determine this by replacing reagent A with C in step 3.

ASSAY FOR ACETYL GROUPS [19]

REAGENTS:

(A) 2.0 M hydroxylaminehydrochloride, mol. wt. = 69.49
(B) 3.5 N NaOH
(C) HCl, 1 part concentrated HCl + 2 parts water
(D) 0.37 M $FeCl_3$ in 0.1 N HCl
(E) Standard = beta-D(+)-glucose pentaacetate, 0.5 mg/ml of MeOH

PROCEDURE:

1) Mix equal parts of reagents A and B just before using. This mixture is stable for 3 hours at room temperature.

2) Prepare 0.4 ml of each sample in separate test tubes. Use MeOH to bring the volume up to 0.4 ml if necessary.

3) Add 0.8 ml of the reagent prepared in step 1 to each tube. Vortex and let stand for at least 1 min.

4) Add 0.4 ml of reagent C to each tube. Vortex.

5) Add 0.4 ml of reagent D to each tube. Vortex.

6) Measure the absorbance at 540 nm.

NOTES:

1. Correction for any non-specific color may be made by repeating assay but reversing the order of reagents C and 1.

2. If samples contain protein in significant quantities, they should be treated with reagent C to precipitate proteins.

PROCEDURE FOR STAINING POLYSACCHARIDE

PROCEDURE:

1) Remove gel and soak it overnight in 40% ethanol (EtOH):5% glacial acetic acid (HOAc):55% deionized water (dH_2O) solution. For 200.0 ml;

80.0 ml EtOH
10.0 ml HOAc
110.0 ml dH_2O

2) Soak gel in oxidizing solution, 10.7% HIO_4 in 40% EtOH:5% HOAc:55% dH_2O, for 5 min. For a 200.0-ml solution;

80.0 ml EtOH
10.0 ml HOAc
110.0 ml dH_2O
1.5 g ($NaIO_4$)

3) Wash gel in dH_2O three times for 15 min each.

4) Soak gel in staining solution (silver nitrate) for 10 min. For 300.0 ml of the staining solution;

56.0 ml 0.1 N NaOH
230.0 ml dH_2O
4.0 ml concentrated ammonium hydroxide (NH_4OH)
10.0 ml 20% (w/v) $AgNO_3$

5) Wash gel as in step # 3.

6) Soak gel in developing solution for 2 - 15 min. For a 200.0-ml developer;

0.5 ml 37% formaldehyde (CH_3CHO)
50.0 mg citric acid
200.0 ml dH_2O

7) Soak gel in stop bath for 30 min. For 200.0 ml of the stop bath solution;

200.0 ml dH_2O
0.7 ml HOAc

8) Wash, then soak in dH_2O overnight.

MILD ACID HYDROLYSIS

PROCEDURE:

1) Prepare about 5 ml of a 5.0 mg/ml solution of the sample in a screw top test tube.

2) Add glacial acetic acid (HOAc) to the sample to make 1% HOAc by volume. Seal the tube with Teflon-lined screw cap and heat at 100°C for 1 hour.

3) Cool the tube on ice and centrifuge.

4) Collect the precipitate and suspend in deionized water. Freeze-dry and weigh. This is going to be the chloroform-soluble fraction of the sample.

5) To the supernatant liquid from step 3, add an equal volume of chloroform. Vortex and centrifuge. Repeat twice more. Collect the chloroform layers, blow-dry using a gentle stream of filtered air, freeze-dry and add to step 4.

6) Freeze-dry and weigh the water layer from step 5. This is going to be the water-soluble fraction of the sample.

7) Chromatograph the water-soluble fraction of the sample in a G-50 Sephadex column, collecting 2-ml fractions at a flow rate of 0.2 ml/min.

URONIC ACID REDUCTION
(METHANOLYSIS PROCEDURE)

A. SOLUTIONS

1. 1.0 M hydrochloric acid in methanol (MeOH) -- prepared by adding 0.720 ml of acetyl chloride to 10 ml cold MeOH, dropwise with stirring.

2. 10.0 mg/ml solution of sodium borohydride in 50% EtOH/1.0 M ammonium hydroxide

3. Stock glacial acetic acid (HOAc)

4. 9:1 solution of MeOH:HOAc

5. MeOH, HPLC grade

B. PROCEDURE

1. Place a 1.0 mg/ml solution of the sample with about 250 µg of hexose, as determined by the anthrone assay, in a screw top test tube and add several beads of Rexyn-H+ resin with vortexing. Remove the aqueous layer after one minute and wash the beads twice with 500 µl of deionized water. Combine all aqeuous layer and blow-dry with a gentle stream of filtered air at 45°C.

2. Add 500 µl of solution 1 to the sample. Seal the tube and heat at 80°C for 16 hours. Cool the sample on ice and blow-dry as in step # 1.

3. Add 250 µl of solution 2 to the sample and sonicate for one hour. Let stand at room temperature overnight.

4. Add 50 µl of solution 3 and repeat twice.

5. Add 500 µl of solution 4 and blow-dry as in step # 1. Repeat three times.

6. Add 500 µl of solution 5 and blow-dry as in step # 1. Repeat three times.

7. Add 20 µl of inositol to the sample and acetylate (usual procedure), starting with the trifluoroacetic acid (TFA) hydrolysis.

MODIFIED BERGERSEN'S MEDIA PREPARATION

INGREDIENT	AMOUNT/LITER
1) Stock Solution # 1	10.0 ml
2) Stock Solution # 2	10.0 ml
3) Stock Solution # 3	10.0 ml
4) Stock Solution # 4	10.0 ml
5) Stock Solution # 5	10.0 ml
6) Yeast Extract	0.5 g
7) Glutamic Acid	0.5 g
8) Mannitol	10.0 g
9) Trace Elements	1.0 ml
10) Agar (added to make solid medium only)	15.0 g

NOTE: pH must be adjusted to 6.8 before autoclaving the media

STOCK SOLUTIONS	AMOUNT/LITER
#1) $CaCl_2.H_2O$	5.0 g
#2) $Na_2HPO_4.12H_2O$	36.0 g
#3) $MgSO_4.7H_2O$	8.0 g
#4) $FeCl_3$	0.3 g
#5) Thiamine.HCl	0.2 g
Biotin	0.2 g

TRACE ELEMENTS (AMOUNT/LITER)

1) $CaCl_2.2H_2O$ (6.6 g)	2) H_3BO_3 (0.145 g)
3) $FeSO_4.7H_2O$ (0.125 g)	4) $CaCl_2.6H_2O$ (0.059 g)
5) $CuSO_4.5H_2O$ (0.005 g)	6) $MnCl_2.4H_2O$ (0.0043 g)
7) $ZnSO_4.7H_2O$ (0.108 g)	8) Na_2MoO_4 (0.125 g)

NOTE: Add EDTA (7.0 g) after adjusting pH to 5.0.

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PHYSIOLOGICALLY BUFFERED SALINE SOLUTION

INGREDIENTS	AMOUNT/LITER
1) KH_2PO_4	0.43 g
2) Na_2HPO_4	1.68 g
3) NaCl	7.20 g

NOTE: Adjust pH to 7.2 prior to use.
This solution is used to extract CPS in a Waring blender from the bacterial pellets.